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High Levels of HIV-1 Replication Show a Clear Correlation With Downmodulation of Bcl-2 Protein in Peripheral Blood Lymphocytes of HIV-1-Seropositive Subjects

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Peripheral blood lymphocytes (PBLs) from 51 HIV-1-seropositive subjects with different levels of HIV-1 replication and 20 healthy blood donors were examined for the expression of the anti-apoptotic Bcl-2 protein. All the plasma samples from HIV-1 patients were characterized for the presence of HIV-1 p24 and HIV RNA viral load. Bcl-2 protein expression in fresh peripheral blood lymphocytes was studied by different tests, including Western blot and indirect immunofluorescence techniques. Direct immunofluorescence staining, revealed by flow cytometry, was applied to quantify the number of specific anti-Bcl-2 antibody epitope binding sites, thus extrapolating the relative number of Bcl-2 into the cells. The results indicate that the expression of Bcl-2 protein is significantly lower in peripheral blood lymphocytes of HIV-1-seropositive patients showing high levels of viral replication, detected by means of HIV-1 p24 and RNA viral load, with respect to HIV-1 patients with low levels of virus replication and healthy blood donors. The clear-cut inverse correlation between viral replication and Bcl-2 expression reinforces the view that HIV-1-mediated apoptosis probably represents a key mechanism in AIDS pathogenesis. ***J. Med. Virol. 56:66–73, 1998.***

KEY WORDS: HIV-1-infected subjects; Bcl-2; peripheral blood lymphocytes; viral load

INTRODUCTION

The role of apoptosis or programmed cell death in the course of several viral infections has received particular attention during recent years [Cohen et al., 1992; Meyaard et al., 1992; Razvi and Welsh, 1995; Revilla et al., 1997]. Programmed cell death has been implicated as a cause of the progressive loss of $CD4^+$ lymphocytes and immune system impairment in HIV-1-seropositive patients [Groux et al., 1992; Meyaard et al., 1992; Muro-Cacho et al., 1995]. Moreover, the apoptotic process is the main mechanism accounting for the human hematopoietic progenitor cell ($CD34^+$) imbalance in HIV-1-infected patients [Re et al., 1993, 1994], in the absence of a productive and/or latent infection [Zauli et al., 1994, 1996].

Since apoptotic changes are not limited to CD4-positive lymphocytes [Finkel et al., 1995; Muro-Cacho et al., 1995], several mechanisms, such as cytokine imbalance [Mangan et al., 1992; Clerici et al., 1994], effects of soluble products [Okada et al., 1997], the extent of viral replication and perhaps the virus strain [Laurent-Crawford et al., 1991; Terai et al., 1991; Martin et al., 1994], have been implicated in the induction of apoptosis.

The products of several cellular genes, such as *c-myc, c-fos, p53, Rb, Fas,* and *Bcl-2,* are involved in the regulation of programmed cell death [Vaux, 1993; Razvi and Welsh, 1995]. Among protooncogenes, *Bcl-2* and its product function as a repressor of apoptosis in multiple cell types, acting at the "effector" stage of apoptosis [Hockenberry et al., 1991; Fairbairn et al., 1993; Kroemer, 1997].

It has been shown that downregulation of Bcl-2 induces a plethora of apoptosis-associated changes, including alterations of the cellular redox state, plasma membrane changes, changes in subcellular ion distribution, and disruption of mitochondrial membrane

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function, with release of protease activators [Kroemer et al., 1995, 1997; Liu et al., 1996; Kluck et al., 1997].

Since the role of apoptosis [Ameisen, 1994; Pantaleo and Fauci, 1995] in the pathogenesis of AIDS still needs to be clearly established, we investigated the expression of Bcl-2 by analyzing the specific anti-Bcl-2 antibody binding sites in peripheral blood lymphocytes obtained from HIV-1-seropositive patients compared with healthy blood donors.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were collected from 51 adult HIV-1-seropositive subjects (32 man and 19 women, aged from 18 to 31 years, mean age 24 ± 5) and 20 HIV-1-negative blood donors, sex- and age-matched, who gave their informed consent to this research according to the Helsinki declaration. For 42 out of 51 HIV-1-seropositive subjects, data concerning the percentage and the absolute number of CD4 and CD8 T-lymphocytes count were also available.

Sample Preparation

Blood samples, collected in EDTA tubes, were processed within 3 hr from blood taking. The plasma was separated from the cell fraction by centrifugation at 700 × g for 10 minutes and then frozen at −70°C until tested for p24 antigen detection and HIV-1 RNA viremia.

Blood samples were layered over Ficoll-histopaque (d = 1.077 g/ml, Pharmacia, Uppsala, Sweden) and centrifuged at 1,500 rpm for 30 min. Light-density mononuclear cells were collected, washed twice in PBS, depleted of monocytes/macrophages by two successive steps of 1 hr adherence in plastic flasks at 37°C with Iscove-modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) plus 10% FCS, and analyzed for the presence of Bcl-2 and tubulin protein expression.

Antigen Detection and HIV RNA Viremia

HIV-1 p24 antigenemia, after immune complex dissociation basic treatment, was evaluated by a commercial enzyme-linked immunosorbent assay kit for specific quantitative determination of HIV-1 *gag* p24 (Organon Teknika Corporation, Durham, NC) according to the manufacturer's instructions. The concentration of HIV-1 p24 antigen for each positive sample was assessed by interpolation from the standard curve by quadratic regression methods. Plasma samples with p24 levels below 3 pg/ml were considered negative.

The RNA quantification was carried out by NASBA (nucleic acid sequence-based amplification) kit according to the manufacturer's instructions (Organon Teknika, Boxtel, The Netherlands). Briefly, 100 μl of plasma was mixed with lysis buffer (guanidine thiocyanate 5 mol/l, Triton X-100, Tris-HCl), and after centrifugation, three synthetic RNA calibrators (named Qa, Qb, and Qc and containing 10^6, 10^5, and 10^4 RNA molecules, respectively) were added to the lysis buffer containing the released nucleic acid. All RNAs were bound to silica particles (solid phase), washed several times by centrifugation, and eluted from the solid phase with 50 μl of Tris-HCl (elution buffer).

Five microliters of the extracted nucleic acid solution were then amplified using a 20 μl reaction mixture, containing 40 mM Tris, 12 mM $MgCl_2$, 42 mM KCl, 5 mM DTT, 15% v/v DMSO, 1 mM each dNTP, 2 mM each NTPs, 0.1 μg/ml BSA, 0.1 U RNAsi H, 40 U T7-RNA polymerase, 8 U AMV-RT (reverse transcriptase), and 0.2 μl of each primers. Amplification of the HIV-1 gag site with the gag-1 primer set, containing the T7 promoter sequence and primer 2, was achieved at 41°C for 90 min.

The amplified wild-type RNA sequence and calibrator RNAs were each quantified with different electrochemiluminescent (ECL) probes in the NASBA QR system for ECL detection, providing a competitive internal linear standard curve based on three values obtained from the three different calibrator RNAs (Qa, Qb, and Qc) used to estimate the patient HIV-1 RNA concentration. The detection limit of isothermal target amplification is 4,000 copies HIV RNA/ml.

Western Blot Analysis of Bcl-2 Protein Expression

Gel electrophoresis was carried out as described by Laemmli [1970]. Briefly, cell lysates were obtained by sonicating 10^6 PBLs cells for 2 min and boiling for 5 min in 62.5 mmol/L Tris buffer, pH 6.8, containing 2% SDS, 5% β-mercaptoethanol, and 10% glycerol. Samples derived from 2×10^6 viable PBLs containing approximately 100 μg of proteins were separated in 12% of acrylamide gel and blotted onto nitrocellulose filters. Blotted filters were blocked for 30 min in a 3% solution of dried skimmed milk in phosphate-buffered saline (PBS) and incubated overnight at 4°C with a 1:100 dilution of anti-Bcl-2 monoclonal antibody (Dako) in 3% milk PBS. Filters were washed in PBS and further incubated for 1 hr at room temperature with 1:2,000 peroxidase-conjugated antimouse IgG (Dako) in 0.5% bovine serum albumin in PBS. Specific reactions were revealed by means of ECL Western blotting detection reagent (Amersham, Arlington Heights, IL). As previously described [Zauli et al., 1995], the same lot of Western blot was stripped and replaced with an anti-tubulin monoclonal antibody (Boehringer) (1:500 dilution) to equalize the lane protein content.

Flow Cytometry Analysis of Bcl-2 Protein Expression

For indirect immunofluorescence technique, 10^6 peripheral blood lymphocytes were fixed in 1% paraformaldehyde in phosphate-buffered saline for 30 min at 4°C, washed twice with PBS, and permeabilized with 0.5% Triton X100 (Sigma, St Louis, MO) in PBS for 10 min at 4°C. After two washings with PBS, cells were resuspended in PBS plus 10% of normal goat serum for 10 min at room temperature before adding anti-Bcl-2 monoclonal antibody (1:50; Dako, Copenhagen, Denmark) and incubated for 1 hr at 4°C. After two wash-

ings in PBS, a policlonal goat antimouse (1:100) antibody conjugated to fluorescein (FITC) (Dako) was added to cells and incubated for 30 min at 37°C. The cells were washed twice and then analyzed by a FACScan flow cytometer (Becton-Dickinson, Palo Alto, CA). In order to assess the specificity of Bcl-2 protein expression, we also evaluated, as a control, the expression of tubulin housekeeping protein by an antitubulin monoclonal antibody (1:50; Boehringer Mannheim, Mannheim, Germany) using the same flow cytometry procedure.

The cells were gated for forward scatter (FSC) and side scatter (SSC) in order to rule out cell aggregates and debris. The negative controls consisted of an isotype-matched unreactive antibody (1:50 dilution of monoclonal antibody directed to human cytomegalovirus late protein p66; Du Pont, Wilmington, DE) followed by an identical second-layer labeling as above.

To quantify cell-associated immunofluorescence, a direct immunofluorescence assay was carried out as previously described [Zauli et al., 1994]. The samples were collected, fixed, and permeabilized as above. After this treatment, the cells were resuspended in PBS plus 10% normal goat serum for 10 min at room temperature before adding either FITC-conjugated anti-Bcl-2 monoclonal antibody (1:50; Dako) or FITC-conjugated anti-CD41 monoclonal antibody (1:50; Dako) as negative control, and incubated for 1 hr at 4°C. The samples were washed in PBS and analyzed by flow cytometry.

FACScan was calibrated before assay by quantitative fluorescein-conjugated beads (Flow Cytometry Standards Corp., Research Triangle Park, NC) containing 0, 1.8×10^4, 8.7×10^4, 1.6×10^5, and 2.4×10^5 fluorescein molecules. A reference curve was constructed by regression analysis after plotting the flow cytometric mean fluorescence intensity value of quantitative FITC-conjugated beads against the log of FITC molecule per beads. Anti-Bcl-2 antibody binding sites were calculated by comparison between the mean fluorescence intensity of the samples and the mean fluorescence intensity of the beads in the reference curve. Relative anti-Bcl-2 monoclonal antibody binding sites per cell were finally achieved by dividing the FITC molecules bound per cell by the fluorescein-to-protein ratio of FITC-conjugated anti-Bcl-2 monoclonal antibody and then by subtracting from this value the number of binding sites obtained by using an isotype-matched control represented by FITC-conjugated anti-CD41 monoclonal antibody. All experiments were carried out under saturation conditions. Therefore, this technique, readily extrapolates the number of intracellular Bcl-2 molecules. Data were expressed as mean of Bcl-2 molecules obtained for each sample, undertaken in triplicate.

Statistical Analysis

The difference in the number of RNA copies per ml of plasma between the two groups of HIV-1-seropositive patients was evaluated by the Mann-Whitney test, a nonparametric statistical procedure based on the sums of ranks.

Statistical comparison of anti-Bcl-2 antibody binding sites in peripheral lymphocytes between the two groups of HIV-1-positive patients and a control group of healthy subjects was made by one-way analysis of variance using for all pairwise multiple comparisons the Bonferroni t-test and the Student-Newman-Keuls q-method.

The relationship between the number of Bcl-2 molecules and the concentration of HIV-1 p24 and the RNA concentrations or the $CD4^+$ and $CD8^+$ lymphocytes count for the samples of HIV-1-positive patients was evaluated by the calculation of the Spearman rank order correlation coefficient (r_s). A partial correlation analysis between the number of Bcl-2 binding sites and the log RNA viral load was made by correcting the correlation factor for the effect of control variables.

All mean values are arithmetical means if not otherwise specified. All the *P* values are two-tailed and a *P* value of 0.05 or less was considered significant. For the statistical analysis, the SPSS statistical software package (Spss Inc., Chicago, IL) was used.

TABLE I. HIV-1 p24 and Viral Load in HIV-1-Seropositive Patients and Healthy Donors[a]

	HIV-1-seropositive patients		Healthy donors
	Group 1	Group 2	Group 3
Number of subjects	30	21	20
HIV-1 p24	<3 pg/ml	54–360 pg/ml	<3 pg/ml
HIV-1 RNA viral load	41,300[b]	270,000[b]	ND[c]

[a]Subjects enrolled in this study were divided into group 1 (HIV-1-seropositive patients with undetectable level of HIV-1 p24), group 2 (HIV-1 seropositive patients with levels of HIV-1 p24 between 54 and 360 pg/ml), and group 3 (healthy subjects, HIV-1-seronegative).
[b]Geometrical mean.
[c]Not done.

RESULTS

HIV-1 p24 Detection and HIV-1 Viral Load in HIV-1-Seropositive Patients

HIV-1-seropositive patients were first tested for the presence of viral p24 level, carried out after basic immunocomplex dissociation. Patients were divided into two groups on the basis of HIV-1 p24 levels detectable in plasma samples. Thirty subjects (group 1) had undetectable levels of p24 (<3 pg/ml) and 21 (group 2) had a large amount of p24 (ranging from 54–360 pg/ml) in the plasma samples. As controls, the plasma from the 20 HIV-1-seronegative blood donors enrolled in this study were also controlled for HIV p24 antigenemia and, as expected, all samples gave negative results (Table I).

Plasma samples were also analyzed for the amount of RNA copy numbers per ml of plasma by the NASBA system. Among the 30 HIV-1-seropositive patients with undetectable levels of HIV-1 p24, 14 showed RNA viral load <4,000 copies/ml and 16 a variable level of HIV RNA between 8,000 and 270,000 (geometrical

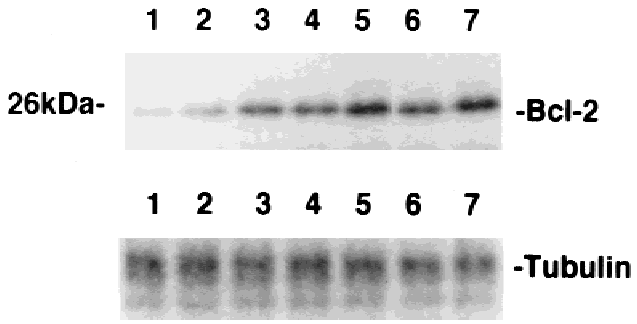

Fig. 1. **Top:** Western blot analysis of BCL-2 protein expression in cellular lysates of PBLs from two patients (No. 2 and 12 of Table II) representative of group 2 (Lane 1, 2); two patients (No. 5 and 24 of Table II) representative of group 1 (Lane 3, 4); and three patients (No. 10, 11, and 12 of Table II) representative of group 3 (control group) (Lane 5, 6, 7). **Bottom:** the level of tubulin expression in some samples belonging to all three groups examined as control of the same Western blot procedure.

mean, 41,300). On the other hand, patients of group 2 with high levels of p24 (between 54 and 360 pg/ml) showed viral loads ranging from 51,000 and 3,600,000 copies/ml (geometrical mean, 270,000) (Table I). As expected, the number of RNA HIV-1 copies, evaluated by the Mann-Whitney test, showed a significant difference ($p < 0.001$) between the two groups considered.

Bcl-2 Antigen Expression in PBLs From HIV-1-Seropositive Patients (Groups 1 and 2) and Healthy Subjects (Group 3)

In order to evaluate Bcl-2 protein expression in fresh peripheral blood lymphocytes obtained from HIV-1-seropositive patients, Western blot assay and an indirect immunofluorescence staining, revealed through a flow cytometric procedure, were carried out.

During Western blot analysis, a higher amount of Bcl-2 protein expression was observed in cellular lysates of peripheral blood lymphocytes obtained from both HIV-1-seropositive patients with a low level of viral replication (group 1) and healthy blood donors (group 3) in comparison with PBL from group 2 of HIV-1-seropositive patients with high levels of HIV-1 p24 and viral load (Fig. 1). On the other hand, the level of tubulin expression, examined as control of the same procedure to equalize the lane protein content, did not reveal any significant differences among the three groups of subjects enrolled in the study.

A parallel set of experiments was carried out using an indirect immunofluorescence staining procedure revealed by flow cytometry (Fig. 2). A lower number of anti-Bcl-2 epitope binding sites was observed in peripheral blood lymphocytes of individuals belonging to group 2 (Fig. 2C) in comparison with PBL obtained from group 1 (Fig. 2B) of HIV-1-seropositive patients and from group 3 (Fig. 2D)

The specificity of Bcl-2 protein downmodulation in the PBL of group 2 patients was confirmed by the parallel analysis of expression of tubulin housekeeping protein in some samples belonging to all three groups. As shown in Figure 3, no significant difference between

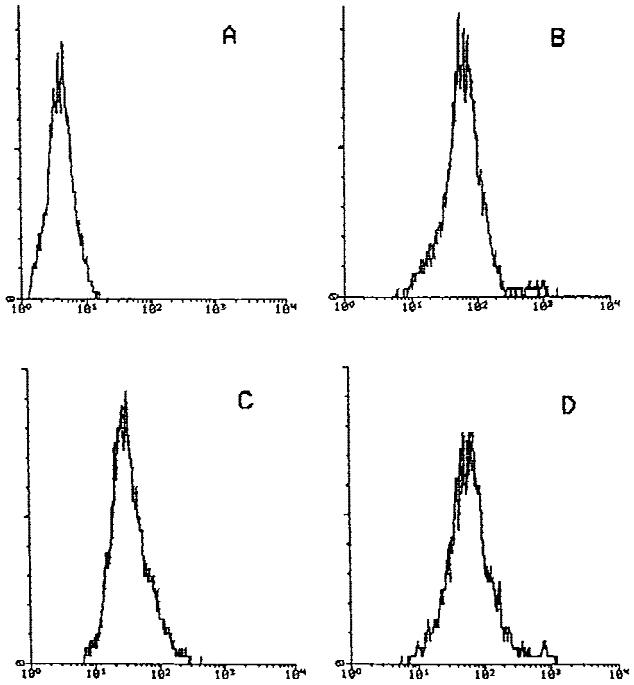

Fig. 2. Bcl-2 protein expression by flow cytometric analysis. BCL-2 expression is shown on lymphocytes detected by indirect staining. X-axis: Green fluorescence intensity of BCL-2 expression detected by fluorescence intensity (logarithmic scale); Y-axis: relative cell number. **A:** Negative control evaluated by means of monoclonal antibody directed to human cytomegalovirus late protein p66 (HCMV p66) and stained with gamig-FITC. **B:** Example of the BCL-2 fluorescence intensity in one patient belonging to group 1. **C:** Example of the Bcl-2 fluorescence intensity in one patient belonging to group 2. **D:** Example of the Bcl-2 fluorescence intensity in one patient belonging to the control group.

the mean fluorescence intensity value in tubulin expression was detected in any of the samples tested.

Number of Bcl-2 Molecules in PBL and Viral Load in HIV-1-Seropositive Patients

Since indirect immunofluorescence is not a quantitative method, a flow cytometry approach was employed to establish the number of anti-Bcl-2 antibody binding sites per cell by means of a direct immunofluorescence assay (Fig. 4).

This analysis confirmed that Bcl-2 protein was less expressed in peripheral blood lymphocytes of HIV-1-seropositive patients with high levels of viral replication detected by the means of HIV-1 p24 and RNA viral load compared with PBLs from patients with low levels of viral replication. In fact, Bcl-2 mean expression per cell was 14,989 (S.D. = 4258) in group 1; 7,920 (S.D. = 1821) in group 2; and 15,531 (S.D. = 4344) in group 3 (Table II).

The one-way analysis of variance showed a statistically significant difference both between Bcl-2 protein mean value of group 1 and group 2 and between group 2 and the healthy control group, whereas no significant difference was observed between group 1 and the control group ($P < 0.05$).

We also determined the relation between the num-

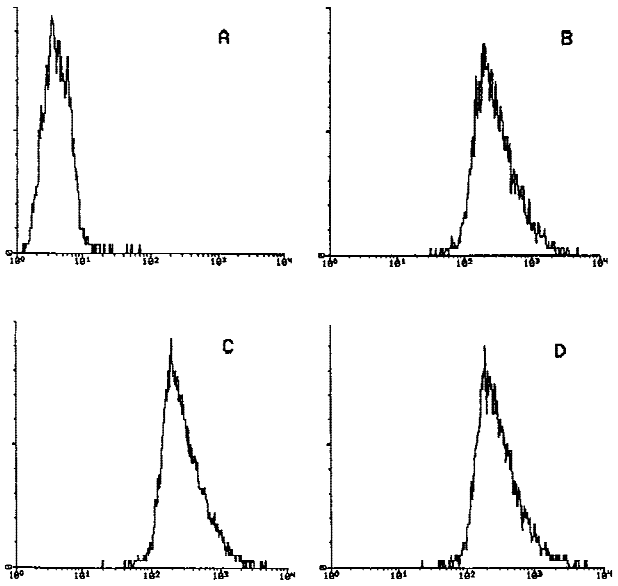

Fig. 3. Analysis of tubulin protein expression by means of indirect immunofluorescence and flow cytometry in PBLs. X-axis: Green fluorescence intensity of Bcl-2 expression detected by fluorescence intensity (logarithmic scale); Y-axis: cell relative number. **A:** nonspecific fluorescence monitored by staining PBLs with monoclonal antibody to HCMV (p66) plus GAMIG-FITC. **B:** Tubulin protein expression in PBLs (group 1) monitored by staining cells with monoclonal antibody to tubulin plus GAMIG-FITC. **C:** Tubulin protein expression in PBLs (group 2) monitored by staining cells with monoclonal antibody to tubulin plus GAMIG-FITC. **D:** Tubulin protein expression in PBLs (group 3) monitored by staining cells with monoclonal antibody to tubulin plus GAMIG-FITC.

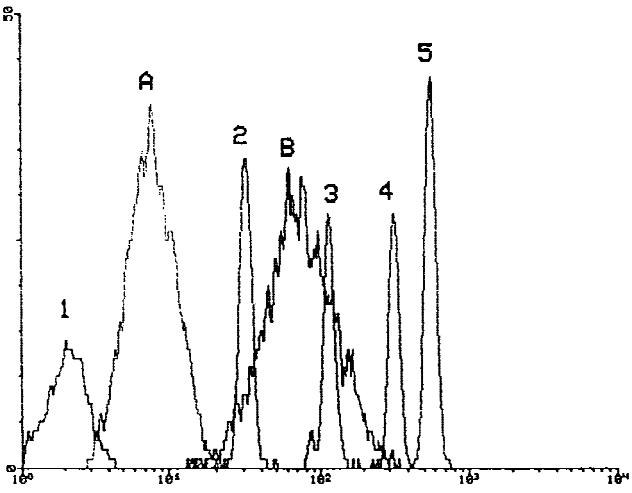

Fig. 4. A typical experiment of Bcl-2 quantification by flow cytometric analysis (direct staining). Relative fluorescence intensity of BCL-2 protein expression on peripheral blood lymphocytes (subjects no. 10—group 1—of Table II) is shown in comparison with quantitative beads. Peaks 1, 2, 3, 4, and 5 represent relative fluorescence of FITC-conjugated beads, whereas peak A represents the negative control achieved with FITC-conjugated anti-CD41 monoclonal antibody and peak B represents the BCL-2 protein expression by direct staining with FITC-conjugated anti-Bcl-2.

bers of anti-Bcl-2 antibody binding sites in PBLs and the concentration of HIV-1 p24 or the RNA concentration in all the plasmas from the 50 HIV-1-positive patients by calculating the Spearman correlation coefficients. A significant negative correlation was found between the Bcl-2 molecule number and the concentration of HIV p24 (r_s = -0.73; $P < 0.001$) and between the number of Bcl-2 molecules and the HIV-1 RNA concentration (r_s = −0.57; $P < 0.001$).

Number of Bcl-2 Molecules in PBL and CD4 and CD8 Counts in 42 HIV-1-Seropositive Patients

We evaluated the correlation between the absolute number of $CD4^+$ and $CD8^+$ cells in 42 HIV-1-seropositive patients. The absolute number of $CD4^+$ was 259 ± 126 in group 1 (range 99–374/μl) and 154 ± 96 in group 2 (range 26–398/μl) and the absolute number of $CD8^+$ cells was 61 ± 15 in group 1 (range 36–82 μl) and 71 ± 7 (range 59–83/μl) in group 2. No significant correlation between the number of anti-Bcl-2 antibody binding sites and the absolute $CD4^+$ lymphocyte count (r_s = 0.18; P = 0.27) or the absolute $CD8^+$ lymphocyte count (r_s = -0.11; P = 0.55) was found. On the other hand, in this group of subjects, we confirmed a significant correlation between the number of anti-Bcl-2 antibody binding sites and the RNA viral load (r_s = −0.48; $P < 0.001$) or p24 concentration (r_s = −0.70; $P < 0.001$).

Moreover, a partial correlation analysis between the number of anti-Bcl-2 antibody binding sites and the log RNA viral load showed that the correlation factor (r = −0.49; $P < 0.001$) was not markedly decreased after removing the effects of absolute $CD4^+$ lymphocyte count (r = −0.48; $P < 0.001$), the percentile $CD4^+$ lymphocyte value (r = −0.45; $P < 0.001$) or the absolute $CD8^+$ lymphocyte count (r = −0.46; $P < 0.001$).

DISCUSSION

Programmed cell death represents an intrinsic cell suicide mechanism induced by a wide array of physiological stimuli. This process is a well-tuned homeostatic mechanism to rule out dysfunctional or dangerous cells, no longer needed by the organism [Kerr et al., 1972; Gerschenson et al., 1992; Vaux, 1993]. Moreover, in viral infection apoptosis is an important pathway of cellular death and has been proposed as a key mechanism to explain $CD4^+$ lymphocyte depletion during the course of HIV-1 infection [Meyaard et al., 1992; Re et al., 1993; Oyaizu et al., 1993; Ameisen, 1994].

In contrast with several gene products that prime cells for apoptosis, others are able to inhibit programmed cell death. Of note is that the first characterized apoptosis-related gene was the *Bcl-2* gene encoding a protein with a powerful anti-apoptotic function [Hockenbery et al., 1990]. This protein is known as a paradigm factor to investigate the cellular survival phenomenon.

It is well known that virus infection and replication are often associated with apoptosis and this effect is likely to be responsible for much of the pathology associated with infectious disease [Young et al., 1997]. Even though some viral infections induce a Bcl-2 overexpression [Klein, 1996; Pillai et al., 1996] in a wide variety of viral diseases, the Bcl-2 expression is dras-

TABLE II. Bcl-2 Protein Expression (Number of Molecules) Revealed by Direct Immunofluorescence Procedure in the Three Groups of Subjects Studied

HIV-1-seropositive patients				Healthy donors	
Group 1		Group 2		Group 3	
Patient number	Bcl-2 number of molecules	Patient number	Bcl-2 number of molecules	Patient number	Bcl-2 number of molecules
1	18,542	1	13,157	1	21,028
2	16,057	2	5,714	2	9,925
3	13,200	3	6,428	3	27,242
4	12,630	4	9,014	4	14,234
5	11,560	5	9,025	5	12,825
6	11,500	6	8,102	6	10,257
7	11,250	7	8,410	7	9,842
8	11,205	8	5,428	8	10,505
9	7,537	9	7,896	9	13,820
10	19,205	10	8,240	10	16,220
11	18,625	11	6,452	11	17,962
12	12,520	12	9,097	12	17,300
13	15,062	13	6,500	13	19,230
14	15,228	14	8,510	14	18,000
15	18,708	15	7,523	15	15,600
16	21,111	16	9,925	16	14,800
17	12,630	17	7,880	17	19,240
18	14,234	18	8,563	18	12,900
19	26,102	19	9,525	19	17,200
20	6,031	20	5,500	20	12,506
21	18,211	21	5,447		
22	13,234				
23	21,297				
24	14,250				
25	12,328				
26	15,400				
27	18,557				
28	12,508				
29	12,577				
30	18,377				
	14,989[a]		7,920[a]		15,531[a]
	4,258[b]		1,821[b]		4,344[b]

[a]Arithmetical Mean.
[b]Standard deviation

tically impaired [Levine et al., 1993, 1996; Tamaru et al., 1993].

Among the additional regulatory factors that have a major influence on viral infection and virus-induced cell death, we focused on the analysis and quantification of Bcl-2 protein expression in the peripheral blood lymphocytes of HIV-1-infected individuals and healthy blood donors. Analysis by Western blot and indirect immunofluorescence showed that the amount of Bcl-2 protein was less expressed in PBL from HIV-1-seropositive patients with a high level of viral replication in comparison with other subjects enrolled in the study. These results were further confirmed by the analysis of the number of specific anti-Bcl-2 antibody binding sites per cell. In fact, a significantly reduced number of epitope binding sites were found in PBLs from HIV-1-seropositive subjects with a high level of HIV-1 p24. These results establish an inverse correlation between the level of HIV-1 replication and Bcl-2 in peripheral blood lymphocytes. This linkage is particularly significant in the presence of high viral loads, suggesting a key role of HIV-1 replication in inducing Bcl-2 downregulation.

Other researchers have investigated the role of Bcl-2 in HIV-1-infected individuals [Boudet et al., 1996] and in cell culture [De Rossi et al., 1994; Park et al., 1996]. Boudet et al. [1996] reported a marked downmodulation of Bcl-2 expression only in a fraction of freshly isolated $CD8^+$ T-lymphocytes, but not in freshly isolated $CD4^+$ T-cell subsets from HIV-1 patients, suggesting that the decrease in Bcl-2 expression in the CD4 subset cannot be observed in vivo probably due to the rapid elimination of these cells from the circulation.

Although our study aimed to verify a Bcl-2 modification in all peripheral blood lymphocytes, the lack of correlation between the number of anti-Bcl-2 antibody binding sites and $CD4^+$ and $CD8^+$ lymphocytes and the partial correlation factor not markedly decreased by controlling for the effects of absolute $CD4^+$ and $CD8^+$ lymphocyte count suggest that the clear decrease of Bcl-2 binding sites observed in HIV-1-seropositive subjects with a high viral replication is not related to variation in the number of different cell subsets considered.

A different conclusion was reached by Park et al. [1996], who failed to find any difference between HIV-

1-infected Jurkat Bcl-2 cells and control cells with respect to the kinetics of virus replication, protein expression, and processing. On the other hand, De Rossi et al. [1994] demonstrated that HIV-1 induced downregulation of Bcl-2 expression in EBV immortalized B-cells.

In addition, Adachi et al. [1996] observed that Bcl-2 expression rapidly decreases in lymphocytes derived from HIV-1-infected patients upon in vitro culture, while this was not observed in cells of healthy volunteers.

Moreover, we cannot rule out that other factors might be involved in apoptosis during the course of HIV-1 disease. Cytokine dysregulation, such as an increased TNF-α, IFN-γ, IL-6, and IL-10 levels and a consistent paucity of IL-2, is one of the characteristic immunological abnormalities associated with HIV-1 infection, and may partly explain the decreased Bcl-2 expression in patients with advanced disease [Emilie et al., 1990; Fan et al., 1993].

In conclusion, our results show that a clear and significant downmodulation of Bcl-2 content was found in HIV-1-infected patients with a high HIV-1 genome copy number, suggesting that monitoring free virus in the blood might be a good prognostic indicator reflecting the relation between the rate of infection and the death of cells. Even though reduced Bcl-2 expression does not appear to be restricted to HIV-1 infection [Tamaru et al., 1989; Akbar et al., 1993] and several mechanisms might be involved in apoptosis during the course of HIV-1 disease, our data suggest that peripheral blood lymphocytes with high levels of viral replication are theoretically more sensitive to the apoptotic process.

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